Cytotoxic α-Halogenoacrylic Derivatives of Distamycin A and Congeners

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The mechanism of action of many antitumor agents involves DNA damage, either by direct binding of the drug to DNA or to DNA-binding proteins. However, most of the DNA-interacting agents have only a limited degree of sequence specificity, which implies that they may hit all the cellular genes. DNA minor groove binders, among which the derivatives of distamycin A play an important role, could provide significant improvement in cancer management, increasing gene specificity, due to high selectivity of interaction with thymine-adenine (TA) rich sequences. We now report and discuss the synthesis, the in vitro and in vivo activities, and some mechanistic features of α-halogenoacrylamido derivatives of distamycin A. The final result of this work was the selection of brostallicin **17** (PNU-166196). Brostallicin, presently in phase II clinical trials, shows a broad spectrum of antitumor activity and an apoptotic effect higher than distamycin derivative tallimustine. An important in vitro toxicological feature of brostallicin is the very good ratio between myelotoxicity on human haematopoietic progenitor cells and cytotoxicity on tumor cells, in comparison with clinically tested DNA minor groove binders. A peculiarity of brostallicin is its in vitro reactivity in the DNA alkylation assays only in the presence of glutathione. Moreover brostallicin's antitumor activity, both in in vitro and in vivo tumor models, is higher in the presence of increased levels of glutathione/glutathione-S-tranferases. These findings contribute to the definition of brostallicin as a novel anticancer agent that differs from other minor groove binders and alkylating agents for both the profile of activity and the mechanism of action and to classify the α-bromoacrylamido derivatives of distamycin as a new class of cytotoxics. Moreover, due to its interaction with glutathione, brostallicin may have a role for the tailored treatment of tumors characterized by constitutive or therapy-induced overexpression of glutathione/glutathione-S-tranferase levels.

Introduction

The biological activity of many low molecular weight antitumor agents appears to be related to their mode and specificity of interaction with particular DNA sequences. Such small molecules are of considerable interest in chemistry, biology, and medicine. Most of the anticancer drugs employed clinically exert their antitumor effect by inhibiting nucleic acid (DNA or RNA) or protein synthesis. Inhibition can occur, for example, through cross-linking of bases in DNA or binding to and inactivation of DNA-related enzymes. Among different classes of cancer chemotherapeutic agents, DNA alkylating compounds have played an important role since the introduction of the nitrogen mustards [*N*,*N*-bis(2-chloroethyl)amines] more than 50 years ago. A drawback common to all DNA alkylating agents is their chemical reactivity. This can result in loss of drug by reaction with other cellular nucleophiles, particularly proteins and low molecular weight thiols, e.g. glutathione (GSH).[1,2] Distamycin A (DST),[3] an antibiotic characterized by an oligopeptidic pyrrolic frame ending with an amidino moiety (Figure 1), binds reversibly to the DNA minor groove with a high selectivity for thymine-adenine (TA)-rich sequences containing at least four TA base pairs.[4,5] Due to the high selectivity of DNA interaction, DST was used in the recent past as a DNA sequence-selective vector of alkylating functions. Tallimustine (TAM), a benzoic acid nitrogen mustard (BAM) derivative of DST (Figure 1), was the result of a drug design rationale which disclosed the possibility of obtaining potent cytotoxic antitumor agents by combining a very mild alkylating moiety, the BAM, with a DNA binding-frame, as in DST, which acts as a sequence-selective vector. This rationale possibly implied the increase of the drug concentration near the DNA avoiding as much as possible aspecific alkylation of biological

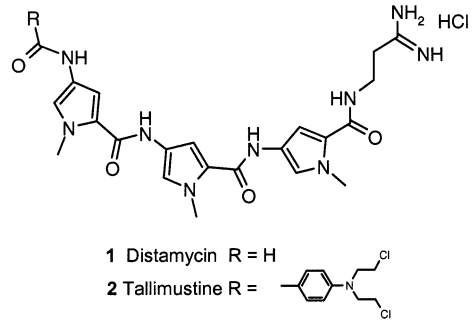

Figure 1. Structure of distamycin A and tallimustine.

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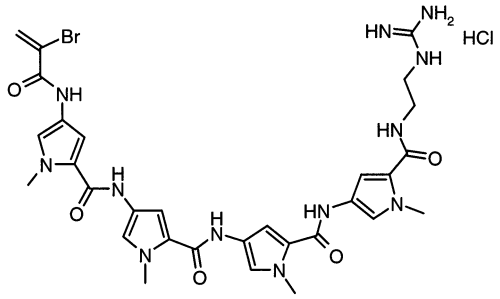


Figure 2. Structure of brostallicin (PNU-166196).

nucleophiles, either proteins or low MW thiols such as GSH, limiting toxicity and drug inactivation, respectively.

TAM selectively alkylates the same DNA sequence recognized by DST[6,7] and shows excellent antitumor activity in preclinical models.[8] TAM was the first and, until very recently, the sole DST-derived cytotoxic agent that was clinically developed for tumor treatment. However, TAM showed a severe dose-limiting myelotoxicity that probably prevented the drug reaching effective therapeutic doses in patients, and its clinical development was discontinued.[9−11] Nevertheless, TAM has represented an important tool for the design of new DNA minor groove binding (MGB) alkylating agents, and a number of nitrogen mustard derivatives of DST or DST-like frames have been reported by several authors.[12−14] Following the same chemical rationale that led to TAM and, taking advantage of the SAR knowledge derived from TAM and congeners, we designed and investigated a new class of compounds characterized by the presence of a α-halogenoacrylic moiety linked to DST or a DST-like frame. We now report the synthesis, in vitro and in vivo activities and some mechanistic features of this new class of agents that led to the selection of brostallicin **17** (PNU-166196) (Figure 2), a potent anticancer agent undergoing phase II clinical trials.

Chemistry

Compounds **3**−**27** were prepared by coupling the acid derivatives **28**−**35** with the suitable amines **36**−**52** as depicted in Scheme 1.

Coupling reactions were carried out either following method A, in which the acids were first activated as acyl chloride derivatives and then coupled with the amines in the Schotten−Baumann conditions using dioxane/water as the solvent or, alternatively, following method B using DCC as condensing agent and THF/water as a solvent. In both procedures $NaHCO_3$ as a base was used. Tripyrrole amine intermediates **36**−**44** were synthesized directly from distamycin A (Scheme 2).

Amide **44** was synthesized, hydrolyzing DST under acidic conditions and then subjecting the intermediate *N*-desformyl-distamycin dihydrochloride **36** to an alkaline hydrolysis in CH_3CN/water at 70°C. Intermediates **37**−**43** were prepared by reacting DST with the appropriate amines **a**−**f** or, in the case of preparation of nitrile **59,** reacting DST with succinic anhydride **g** and K_2CO_3 in DMF, following a procedure previously reported by us,[15] and then treating the corresponding intermediates **53**−**59** with HCl at room temperature. The reactions for the preparation of compounds **53**−**56** were carried out in DMF at room temperature in a range of time from 2 to 72 h. In the case of **56**, the addition of sodium hydride as a base was required while, in case of compound **57**, it was necessary to raise the temperature to 80 °C. The synthesis of compound **58** gave the best results when methanol was used as the solvent. For the synthesis of amino-guanidine derivatives **45**−**47**, amino-modified guanidine derivatives **48**, **49**, the urea derivative **50,** and the amidino derivatives **51** and **52**, a step by step total synthesis was required (Scheme 3).

1-Methyl-4-nitropyrrole acid chloride **60** was reacted with the amines **95**−**101** under classical Schotten−Baumann conditions. The nitro functionality of intermediates **61**−**66** was reduced catalytically using 5% Pd/C, 2 N HCl, under 40 psi pressure in methanol/water (1/1, v/v) as the solvent, while for the intermediate **67**, $NaBH_4$ as reducing agent was used. The length of the polypyrrole frame was increased through a series of acylation/reduction steps until the desired intermediates were reached. For the synthesis of compound **50**, the precursor **93** was first converted in the corresponding amido derivative **94** by removal of the Boc protecting group followed by reaction with 1,1′-carbonyldiimidazole and then treatment with ammonia. Finally the nitro group of intermediate **94** was reduced by treatment with 5% Pd/C, 2 N HCl using the same procedure reported above for the reduction of compounds **61**−**66**. Amino intermediates **95** and **100** were synthesized according to the literature,[16,17] while compound **101** and its congeners **102** and **103** which are all commercially available, were used for the preparation of intermediates **96**−**99** as depicted in Scheme 4.

The amino group of the commercially available compounds **101**−**103** was first replaced by a guanidino or a guanidino-modified group using 2-methyl-isothiourea or 1,2,3-trimethyl-isothiourea, respectively, in refluxing EtOH as a solvent for 8−10 h, and then the Boc protecting group was removed with HCl/methanol to give the final compounds with 60−70% overall yield.

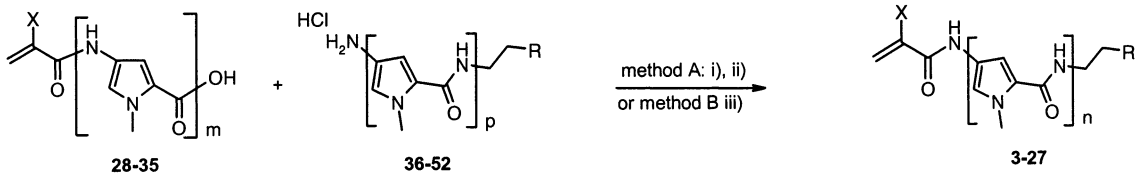
Results and Discussion

Modifications of the Acrylamido Moiety. Table 1 summarizes the in vitro cytotoxicity and in vivo antitumor activity of DST derivatives modified at the acrylic moiety.

While α-bromo and α-chloro compounds **3** (PNU-151807) and **4** show relevant and similar in vitro cytotoxic activity and an in vivo antitumoral activity, fluoro analogue **5** and the simple acrylamido derivative **6** appear devoid of cytotoxity. These data suggest the key role of the reactivity of the α-halogenoacrylic moiety for cytotoxicity. We hypothesized that the chemical reactivity of the α-acrylic moiety could be based on a Gabriel−Cromwell-like reaction,[18,19] wherein a Michael addition from a nucleophile to the double bond gave a reactive α-halogenoamido intermediate which can easily react with a second nuclophile, e.g., under biological conditions, the DNA nucleophilic functions.

To verify this hypothesis, α-bromo-, α-chloro-, and α-fluoroacrylic model compounds were prepared and their chemical reactivity versus nitrogen nucleophiles was evaluated. Scheme 5 summarized the chemical reactivity of the 4-(2-bromoacryloylamino)-1-methyl-1*H*-

Scheme 1[a]



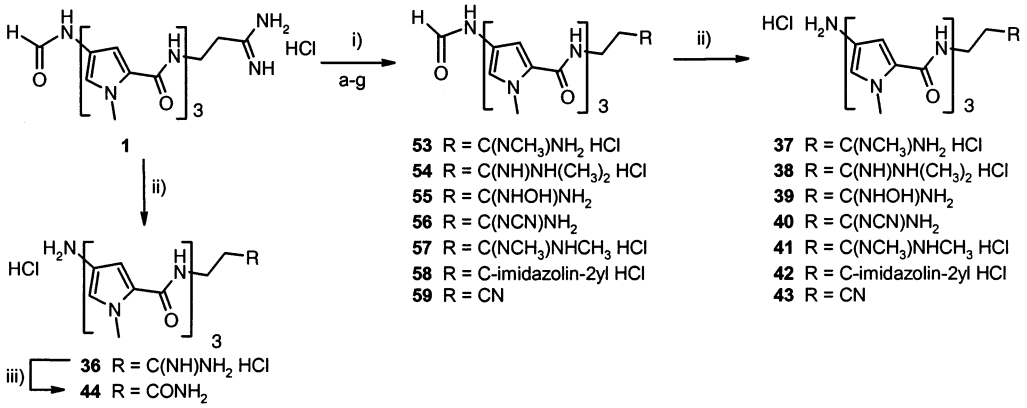
28 X = Br, m = 0
29 X = Cl, m = 0
30 X = F, m = 0
31 X = Br, m = 1
32 X = Cl, m = 1
33 X = F, m = 1
34 X = H, m = 1
35 X = Br, m = 2

36 R = $C(NH)NH_2$ HCl	P = 3	
37 R = $C(NCH_3)NH_2$ HCl	P = 3	
38 R = $C(NH)NH(CH_3)_2$ HCl	P = 3	
39 R = $C(NHOH)NH_2$	P = 3	
40 R = $C(NCN)NH_2$	P = 3	
41 R = $C(NCH_3)NHCH_3$ HCl	P = 3	
42 R = C-imidazolin-2yl HCl	P = 3	
43 R = CN	P = 3	
44 R = $CONH_2$	P = 3	
45 R = $NHC(NH)NH_2$ HCl	P = 3	
46 R = $CH_2NHC(NH)NH_2$ HCl	P = 3	
47 R = $(CH_2)_2NHC(NH)NH_2$ HCl	P = 3	
48 R = $NHC(NCH_3)NHCH_3$ HCl	P = 3	
49 R = NHC-imidazolin-2yl HCl	P = 3	
50 R = $NHCONH_2$	P = 3	
51 R = $C(NH)NH_2$ HCl	P = 2	
52 R = $C(NH)NH_2$ HCl	P = 1	

			Method
3 R = $C(NH)NH_2$ HCl	X = Br	n = 4	A
4 R = $C(NH)NH_2$ HCl	X = Cl	n = 4	B
5 R = $C(NH)NH_2$ HCl	X = F	n = 4	A
6 R = $C(NH)NH_2$ HCl	X = H	n = 4	A
7 R = $C(NH)NH_2$ HCl	X = Br	n = 5	A
8 R = $C(NH)NH_2$ HCl	X = Br	n = 3	B
9 R = $C(NH)NH_2$ HCl	X = Br	n = 2	A
10 R = $C(NH)NH_2$ HCl	X = Br	n = 1	B
11 R = $C(NH)NH_2$ HCl	X = Cl	n = 3	B
12 R = $C(NH)NH_2$ HCl	X = F	n = 3	A
13 R = $C(NCH_3)NH_2$ HCl	X = Br	n = 4	A
14 R = $C(NCH_3)NHCH_3$ HCl	X = Br	n = 4	A
15 R = $C(NH)NH(CH_3)_2$ HCl	X = Br	n = 4	A
16 R = C-imidazolin-2yl HCl	X = Br	n = 4	A
17 R = $NHC(NH)NH_2$ HCl	X = Br	n = 4	A
18 R = $NHC(NH)NH_2$ HCl	X = F	n = 4	A
19 R = $NHC(NCH_3)NHCH_3$ HCl	X = Br	n = 4	A
20 R = $CH_2NHC(NH)NH_2$ HCl	X = Br	n = 4	A
21 R = $(CH_2)_2NHC(NH)NH_2$ HCl	X = Br	n = 4	A
22 R = NHC-imidazolin-2yl HCl	X = Br	n = 4	A
23 R = $NHCONH_2$	X = Br	n = 4	A
24 R = $C(NHOH)NH_2$	X = Br	n = 4	A
25 R = $C(NCN)NH_2$	X = Br	n = 4	A
26 R = $CONH_2$	X = Br	n = 4	A
27 R = CN	X = Br	n = 4	A

[a] Reagents and conditions: (i) toluene, $SOCl_2$, reflux 2 h; (ii) dioxane/water, $NaHCO_3$, rt, 2 h; (iii) THF/H_2O, DCC, $NaHCO_3$, rt, 8−24 h.

Scheme 2[a]



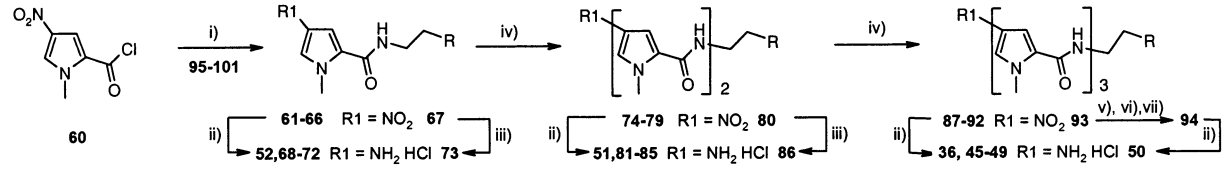
[a] Reagents and conditions: (i) (a) DMF, 80% CH_3NH_2·HCl in water, rt, 12 h; (b) DMF, $(CH_3)_2NH$ hydrate, rt, 72 h; (c) DMF, NH_2OH, 70 °C, 2 h; (d) DMF, NaH, NH_2CN, rt, 3 h; (e) DMF, CH_3NH_2 hydrate, 80 °C, 6 h; (f) MeOH, $NH_2CH_2CH_2NH_2$, rt, 24 h; (g) DMF, succinic anhydride, Na_2CO_3, 70 °C, 3 h; (ii) MeOH, 2 N HCl, rt, 72 h; (iii) CH_3CN/H_2O, NaOH, 70 °C, 4 h.

pyrrole-2-carboxylic acid phenylamide **104** under hydrolytic conditions and with primary and secondary amines under different conditions.

Compound **104** was found to be stable in hydrolytic conditions at pH = 10, while it reacts with primary or secondary amines giving the aziridine derivative **105** or the adduct **106**, respectively, both derived from a first Michael attack followed by halogen substitution. In the case of reaction with weak nucleophiles, such as adenine or imidazole, compound **104** reacts only in the presence of a strong base, giving the adducts **107** and **108** that can be considered derived from a Michael attack followed by *β*-elimination. When the bromine was substituted by fluorine or chlorine, we observed that, in the same or harder reaction conditions, the 4-(2-fluoroacryloylamino)-1-methyl-1*H*-pyrrole-2-carboxylic acid phenylamide did not react, while the 4-(2-chloro-acryloylamino)-1-methyl-1*H*-pyrrole-2-carboxylic acid phenylamide showed an intermediate reactivity. The different reactivity toward nucleophiles between the α-bromo and the α-fluoro moiety on the model compounds is in agreement with the dramatically different cytotoxicity of derivatives **3** and **5**, the former very potent (IC_{50} = 6.3 nM), the latter inactive (IC_{50} > 700 nM). Also the inactivity of acrylic derivative **6** confirms the requirement of a reactive halogen on the double bond.

PNU-151807 was further investigated by DNA footprinting analysis, and it was found to bind reversibly to the same DNA minor groove regions recognized by TAM and DST but was found unreactive in classical in

Scheme 3[a]

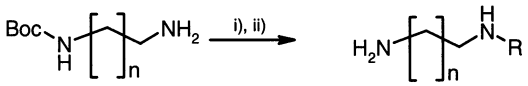


95 $NH_2(CH_2)_2C(NH)NH_2$ 2HCl
96 $NH_2(CH_2)_2NH$-$C(NH)NH_2$ 2HCl
97 $NH_2(CH_2)_2NHC(NCH_3)NHCH_3$ 2HCl
98 $NH_2(CH_2)_3NHC(NH)NH_2$ 2HCl
99 $NH_2(CH_2)_4NHC(NH)NH_2$ 2HCl
100 $NH_2(CH_2)_2$NHC-imidazolin-2-yl 2HCl
101 $NH_2(CH_2)_2NHCOO(CH_3)_3$ HCl

Compound	R
61, 52, 74, 51, 87, 36	$C(NH)NH_2$ 2HCl
62, 68, 75, 81, 88, 45	NH-$C(NH)NH_2$ 2HCl
63, 69, 76, 82, 89, 48	$NHC(NCH_3)NHCH_3$ 2HCl
64, 70, 77, 83, 90, 46	$CH_2NHC(NH)NH_2$ 2HCl
65, 71, 78, 84, 91, 47	$(CH_2)_2NHC(NH)NH_2$ 2HCl
66, 72, 79, 85, 92, 49	NHC-imidazolin-2-yl 2HCl
67, 73, 80, 86, 93	$NHCOO(CH_3)_3$
94, 50	$NHCONH_2$

[a] Reagents and conditions: (i) water/dioxane (1/1, v/v), $NaHCO_3$, rt; (ii) MeOH/dioxane (1/1, v/v), 2 N HCl, H_2, 5% Pd/C, 40 psi, rt; (iii) MeOH/dioxane (3/1, v/v), $NaBH_4$, 0 °C → rt; (iv) water/dioxane (1/1, v/v), **59**, $NaHCO_3$, rt; (v) MeOH/HCl, rt, 2 h; (vi) DMF, TEA, CDI, 0 °C → rt, 3 h; (vii) EtOH, NH_3, rt, 3 h.

Scheme 4[a]

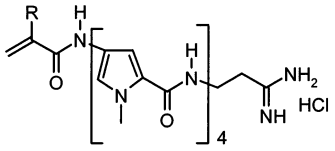


101 n = 1
102 n = 2
103 n = 3

96 n = 1; R = $C(NH)NH_2$ HCl
97 n = 1; R = $C(NCH_3)NHCH_3$ HCl
98 n = 2; R = $C(NH)NH_2$ HCl
99 n = 3; R = $C(NH)NH_2$ HCl

[a] Reagents and conditions: (i) EtOH, $NH_2C(SCH_3)NHCH_3$, or $CH_3NHC(SCH_3)NHCH_3$, reflux; (ii) HCl/MeOH, rt.

Table 1. In Vitro and in Vivo Antitumor Activity of α-Halogenoacrylamido Derivatives



		L1210 murine leukemia[a]		
		in vitro[b]	in vivo[c]	
compd	R	IC_{50} (nM)	O.D. (mg/kg)	T/C%
3	Br	6.3 ± 1.3	1.56	200
4	Cl	3.8 ± 1.4	1.56	133
5	F	>700	n.d.	n.d.
6	H	>3000	n.d.	n.d.

[a] L1210 cells were obtained from NCI, Federick, MD. [b] Drug cytotoxicity was determined after 48 h of continuous exposure by counting surviving cells; IC_{50} = 50% inhibitory concentration as the mean ± SE from dose−response curves of at least six replicates. [c] For in vivo studies L1210 cells were injected iv at day 0 and mice were treated iv the day after tumor inoculum; O.D.= optimal (nontoxic) dose ≤ LD10; T/C% = median survival time of treated vs untreated mice × 100; n.d. = not determined.

vitro DNA alkylation assays,[20] at variance not only with TAM, but also with other cytotoxic MGBs. Moreover, PNU-151807 showed a significantly better cytotoxicity/myelotoxicity ratio in respect to TAM[21] and was fully effective against DNA-mismatch-repair-deficient tumor cells.[22] All these findings contribute to classify the α-bromoacrylamido-DST derivatives as a new class of compounds.

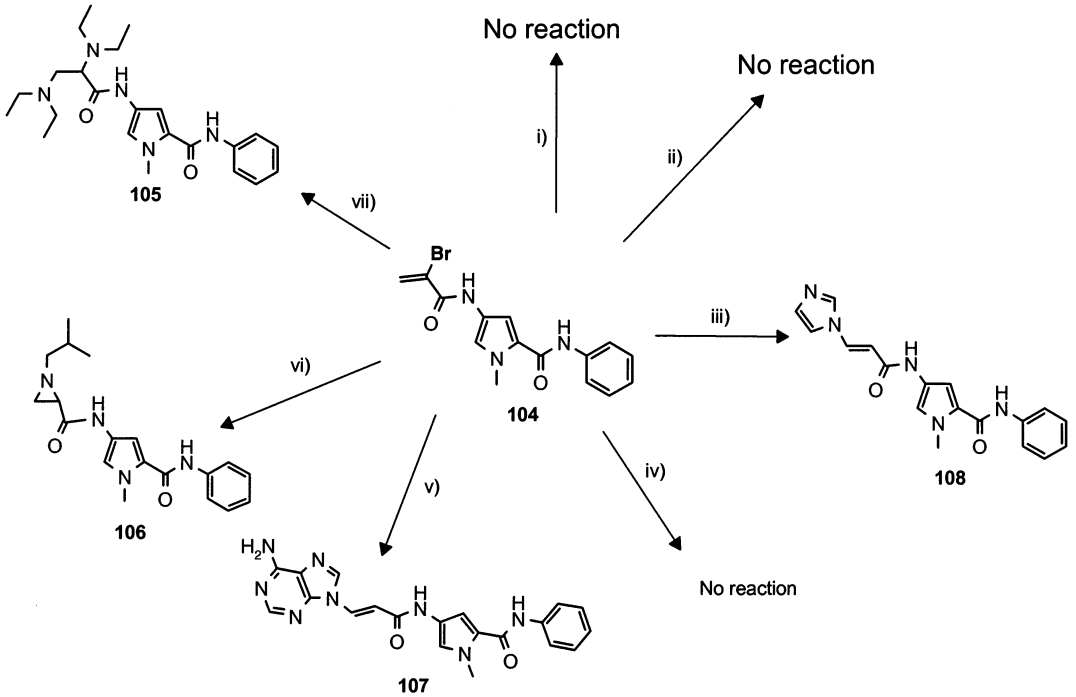
Modifications of Polypyrrole Frame of α-Halogenoacrylamido Derivatives. The unusual mechanistic features shown by PNU-151807, the first lead of the α-bromoacrylamido DST-like class, prompted us to synthesize new halogenoacrylic DST derivatives and congeners with the aim of optimizing their activity profile and contributing to define their mechanism of action. Table 2 reports the in vitro and in vivo activity on tumor cells of α-halogenoacrylamido derivatives of DST modified on the length of the polypyrrole frame.

As far as the in vitro and in vivo data are concerned, the length of the polypyrrole frame plays a role in the potency of this class of compounds. In fact, while both α-bromoacrylamido and α-chloroacrylamido derivatives **8** and **11** of the same DST frame are equipotent in vitro, the four-pyrrole unit derivatives **3** (PNU-151807) and **4** are 1 order of magnitude more cytotoxic than the three-pyrrole unit congeners. The same decrease of activity occurs with compound **9** and **10**, which are therefore devoid of significant cytotoxic activity. Five-pyrrole unit derivative **7** seems not to follow this trend of cytotoxicity. The increasing of activity with the number of pyrrole units is in agreement with the result obtained in the past with TAM and its derivatives and could be due to an increased multiplicity of interactions with the DNA minor groove.[23,24] Increasing the number of pyrroles from three to five leads also to an higher in vivo potency (O.D.mg/kg), while the antitumor effect (T/C%) shows a peak in the case of compound **3** (PNU-151807).

Cell permeability is one of the key players in the in vitro and in vivo activity of drugs. In vitro Caco-2-cells permeability experiments have been used to verify a possible correlation between length of the polypyrrole frame, activity, and permeability for this class of compounds (Table 3).

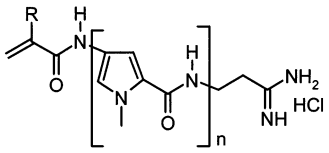
All the compounds in Table 3 are characterized by a negative calculated distribution coefficient (ClogD) that represents a negative factor for passive transport through the cellular membrane.[25] However, Caco-2-cells data show that there is a progressive increase of permeability ($P_{app} \times 10^{-6}$ cm/s) for the compounds containing from one to four pyrrole units, in accordance with their higher cytotoxicity. This apparent contradiction could be explained by the presence of an active transport mechanism able to recognize the DST-like framework when the number of the pyrrole units is up to two and that may be justified by the biotic nature of DST itself. Further evidence for the ability of PNU-151807 to enter

Scheme 5[a]



[a] Reagents and conditions: (i) 1:1 acetone/water, NaOH (pH = 10), 60 °C 48 h; (ii) DMF, imidazole, 80 °C 8 h; (iii)DMF, imidazole, K_2CO_3 (2 equiv), 60 °C 8 h; (iv) DMF, adenine, 12 h; (v)DMF, adenine, K_2CO_3 (1 equiv) 12 h; (vi) DMF, isobutylamine, 70 °C 4 h; (vii) EtOAc, diethylamine, 1 week.

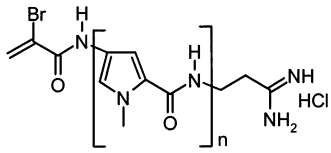
Table 2. In Vitro and in Vivo Antitumor Activity of α-Halogenoacrylamido Derivatives Modified on the Framework



			L1210 murine leukemia[a]		
			in vitro[b]	in vivo[c]	
compd	R	*n*	IC_{50} (nM)	O.D. (mg/kg)	T/C%
7	Br	5	23.0[d] ± 5.0	0.78	167
3	Br	4	6.3 ± 1.3	1.56	200
8	Br	3	98.8 ± 24.2	12.50[e]	175
9	Br	2	1300	n.d.	n.d.
10	Br	1	>2500	n.d.	n.d.
4	Cl	4	3.8 ± 1.4	1.56	133
11	Cl	3	96.8 ± 24.2	12.50	117
5	F	4	>700	n.d.	n.d.
12	F	3	>800	n.d.	n.d.

[a–c] See Table 1, footnotes a–c. [d] Data are referred to 4 h treatment. [e] Data are referred to ip treatment.

Table 3. Caco-2 Cells Permeability of α-Bromoacrylamido Derivatives



		Caco-2		L1210
compd	*n*	Papp × 10^{-6} cm/s[a]	ClogD (pH 7.4)[b]	IC_{50} (nM)[c]
3	4	38.20	−6.63	6.30
8	3	15.21	−6.00	98.80
9	2	5.58	−5.38	1300
10	1	0.69	−4.75	>2500

[a] Apparent permeability coefficient determined after 120 min of incubation according to the method reported by P. Artusson. [b] Values are calculated using ACD LogD software version 6.0 (Advantage Chemistry Development Inc., Toronto, Canada). [c] Drug cytotoxicity was determined after 48 h of continuous exposure against L1210 murine leukemia cells; IC_{50} = 50% inhibitory concentration from dose−response curves of at least six replicates.

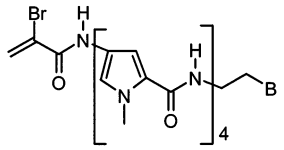
into cells was obtained by measuring its intracellular accumulation and distribution. In vitro experiments with human ovarian carcinoma (A2780) cells show that PNU-151807 is rapidly uptaken into the cells, reaching the steady state in the first hour of exposure, and that about 75% of the drug is localized in the nucleus (data not shown).

Total or partial replacement of the pyrrole units with other heterocyclic nuclei such as pyrazole, imidazole, thiazole, indole, or benzofuran was also investigated.[26–29] The effect of these modifications has in general decreased the antitumor activity or, in the best cases, the compounds did not show a substantial improving of the activity profile.

α-Bromoacrylamido Derivatives Modified at the Amidino Moiety. The role of the basic end terminal of α-bromoacrylamido DST-like derivatives has been investigated, modifying either the basic moiety's strength and the distance of the positive charge from the polypyrrole backbone. In the first series of compounds reported in Table 4, the basic amidino moiety was replaced with groups characterized by an increased or decreased basicity or with groups not basic at all.

Data in the Table 4 show that modifications of the basic strength moiety are not detrimental for the activity of this class of molecules. Compounds keeping a strong basic moiety as the amidino-modified derivatives **13**−**16**, the guanidino derivative **17**, the guanidino modified derivatives **19**, **22**, or compounds not basic at

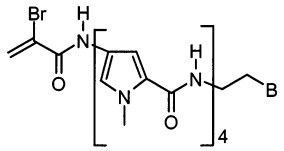
Table 4. In Vitro Cytotoxic Activity of α-Bromoacylamido Derivatives Modified at the Amidino Moiety



compd	B	IC_{50} (nM)[a]
3	$C(NH)NH_2 \cdot HCl$	6.31 ± 1.34
13	$C(NCH_3)NH_2 \cdot HCl$	2.67 ± 0.78
14	$C(NCH_3)NHCH_3 \cdot HCl$	1.86 ± 0.18
15	$C(NH)N(CH_3)_2 \cdot HCl$	1.48 ± 0.84
16	C-imidazolin-2-yl·HCl	2.46 ± 0.52
17	$NHC(NH)NH_2 \cdot HCl$	1.85 ± 0.17
19	$NHC(NCH_3)NHCH_3 \cdot HCl$	1.26 ± 0.38
22	NH-C-imidazolin-2-yl·HCl	3.81 ± 0.89
23	NHCONH2	48.90 ± 9.00
24	$C(NOH)NH_2$	8.58 ± 1.55
25	$C(NCN)NH_2$	4.13 ± 027
26	$CONH_2$	9.00 ± 0.43
27	CN	18.10 ± 2.27

[a] See Table 1, footnotes a and b.

Table 5. In Vitro Cytotoxic Activity of α-Bromoacylamido Derivatives Modified at the Amidino Moiety



compd	B	IC_{50} (nM)[a]
17	$NHC(NH)NH_2 \cdot HCl$	1.85 ± 0.17
20	$CH_2NHC(NH)NH_2 \cdot HCl$	20.70 ± 8.70
21	$(CH_2)_2NHC(NH)NH_2 \cdot HCl$	7.20 ± 3.20

[a] See Table 1, footnotes a and b.

all as the derivatives **24**−**27** showed an in vitro cytotoxicity comparable to that of parent compound **3**. Also for this new class of compounds, as already reported in the case of nitrogen mustard derivatives of DST,[30] the data confirm a lack of correlation between the basicity of the amidino-replacing moiety and cytotoxicity. One exception is represented by the inactive ureido derivative **23**, which shows vs the guanidino **17** the hypothesised structural analogy shown by the active amide **26** vs the amidine **3**.

Table 5 reports a guanidino-restricted series of compounds wherein the distance between the polypyrrole backbone and the positive charge has been evaluated. The data show that in both compounds **20** and **21**, wherein the distance between the positive charge of the guanidino moiety from the polypyrrole frame has been increased by addition of one and two alkyl units, respectively, are less cytotoxic than the progenitor guanidino derivative **17**.

Brostallicin (PNU-166196). As the result of in vitro and in vivo SAR studies performed on a series of α-halogenoacrylic derivatives of DST and DST-like classes, brostallicin was selected for clinical development. Brostallicin, the α-bromoacrylamido-tetrapyrrole-carbamoyl derivative ending with a guanidino moiety (Figure 2), showed an outstanding preclinical activity profile[31] and is presently undergoing Phase II clinical evaluation. An important in vitro toxicological finding for brostallicin was the radically reduced myelotoxicity compared to TAM and other clinically tested MGBs which was observed in patients severe delayed and cumulative myelotoxicity.[9] As reported in Table 6, brostallicin presents a remarkable in vitro therapeutic index, its mean IC_{50} against tumor cell lines being about 80 times lower than its IC_{50} on human CFU-GM hematopoietic progenitors cells.[32] Moreover, brostallicin shows in vitro cytotoxicity and apoptotic effect higher than TAM[33] and a broad spectrum of antitumor activity in in vivo experimental models (Table 7). Brostallicin circumvents resistance to alkylating agents and topoisomerase I inhibitors and, unlike several MGBs, it is active against DNA-mismatch-repair-deficient tumor cells.[34]

Like that of its amidino analogues PNU-151807, brostallicin does not alkylate DNA in in vitro assays.[32] On the basis of the chemical reactivity of this class of compounds, we speculated that an intracellular reactive nucleophilic species, e.g. GSH, could activate the molecule by a first-step Michael-type attack, which may be followed by a further reaction of the no more vinylic bromide, leading to alkylation of DNA nucleophilic functions (Figure 3). Thus, brostallicin might alkylate DNA only in the presence of GSH.

Table 6. Comparison of the in Vitro Myelotoxicity/Cytotoxicity on Tumor Cells Ratio between Brostallicin and Others MGBs

compd	myelotoxicity: human CFU-GM[a] IC_{50} (ng/mL)[c]	cytotoxicity: tumor cells[b] IC_{50} (ng/mL)	therapeutic index[d]
brostallicin	3127	38.54	81.1
tallimustine	395	449	0.9
carzelesin	0.20	0.05	4.0
bizelesin	0.22	0.21	1.0
adozelesin	0.34	0.08	4.2

[a] The myelotoxicity has been tested in vitro on human granulocyte macrophage colony forming units (CFU-GM) cord blood derived hematopoietic cells (1 h exposure). [b] Drug cytotoxicity was determined after 1 h exposure against six different human tumor cells lines. [c] IC_{50} = 50% inhibitory concentration as the mean from dose−response curves of at least six replicates. [d] Therapeutic index = ratio between IC_{50} on human bone marrow progenitors (CFU-GM) and tumor cells.

Table 7. In Vivo Activity of Brostallicin on Human Carcinoma Xenografts

tumor[a]		treatment schedule[b]	optimal dose (mg/kg/day)	%TI[c]
ovarian	A2780	q4d×2	0.78	99
	H207	q7d×4	0.52	100[e]
renal	Caki 2	q7d×3	0.78	84
prostatic	DU 145 early[d]	day 1,5	0.52	77
	DU 145	q7d×3	0.78	67
small cell lung	N592	q7d×3	0.78	72
non small cell lung	A549	q7d×3	0.78	59
colon	HCT-116	q7d×3	0.78	69
	HT-29	q7d×3	0.78	42
pancreatic	Capan 1	q7d×3	0.52	38
gastric	GTL 16	q7d×3	0.78	67

[a] Tumors were transplanted subcutaneously on athymic mice. When the tumor was palpable (0.2−0.3 g), animals were divided randomly into test groups consisting at least of six mice each (day 0). Drug was administered iv in a volume of 10 mg/kg of body weight according to the indicated schedules. [b] Treatment schedules: every 4 days × 2 administrations (q4d×2), weekly × 4 administrations (q7d×4) or × 3 administrations (q7d×3) and administered on day 1 and 5 (day 1,5) [c] % tumor growth inhibition is calculated in respect to controls one week after the end of the treatment. [d] Treatment starts the day after tumor implant. [e] 10% cured mice.

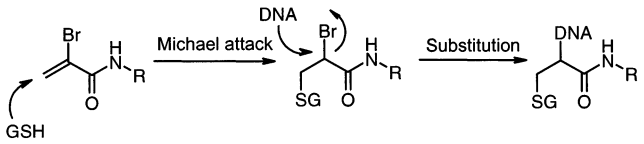


Figure 3. Hypothesized mechanism of activation of α-bromoacrylamido derivatives by GSH.

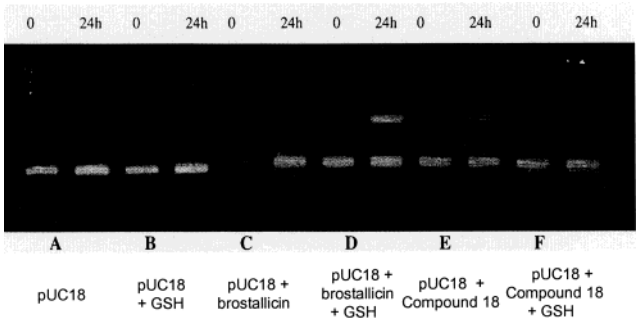


Figure 4. Interaction of brostallicin with plasmidic DNA. Supercoiled pUC18 (9 nM) was incubated at 37 °C for 0 or 24 h (10 mM Tris-acetate/1 mM EDTA buffer pH = 8.0) at final drug concentration of 9 mM. DNA samples were loaded on 0.8% agarose gel in 40 mM Tris-acetate/1mM EDTA buffer pH 7.7, electrophoresis was run at costant 100 V and then stained with ethidium bromide, and finally the DNA bands were revealed by UV light.

Interaction of Brostallicin with GSH. GSH is the most abundant biological thiol, whose concentration is in the millimolar range (0.5−10mM) in mammalian cells and it reaches micromolar concentration in blood plasma.[35] GSH and GSH−S-transferase (GST) family enzymes, which catalyze the reaction of GSH, are involved in several protection mechanisms of cell against reactive oxygen compounds and free radicals or in the detoxification of drugs[36] and represent one of the main mechanisms of drug resistance in tumor cells.[37] GST's may catalyze the conjugation of GSH to electrophilic molecules changing their solubility and excretion properties or, as in the case of alkylating agents, may sequester drugs by direct binding.[38] It is reported that cytotoxic activity of alkylating mustards, chlorambucil (CLB) and melphalan (L-PAM) or platinum derivatives such as cisplatin, is influenced by GSH and GSTs levels.[39] The GSH/GTSs level/expression has been shown to be higher in tumor cells in respect to normal tissues or to be increased following exposure to cytotoxic antitumor drugs. Moreover, an increase in CLB sensitivity with cells pretreated with a GST inhibitor was reported.[40]

Interestingly, on the contrary to that reported for CLB and other MGBs, the GSH/GST system plays a key positive role in the mechanism and activity of brostallicin. The involvement of GSH in the mechanism of action of brostallicin and congeners was supported by several experimental findings. First evidence, aimed at demonstrate the covalent binding between brostallicin and DNA, was shown in nicking experiments using the pUC18 plasmid as model. The covalent adducts are thermally unstable and spontaneously generate nicking of the double strand of DNA with the subsequent relaxation of the plasmid supercoiled form to the circular form, which is highlighted by a different electrophoretic band (Figure 4). This was indeed observed when plasmidic DNA is incubated with brostallicin, but only after the addition of GSH. In the same experimental conditions, mixtures containing only pUC18 or pUC18 and GSH or pUC18 and brostallicin are unable to nick the plasmidic DNA.[41] The inactive fluoroacrylic analogue **18** either in absence or in the presence of GSH does not react covalently with plasmidic DNA.

Table 8. In Vitro Cytotoxicity on L1210 and on L1210/L-PAM Cells

compd	L1210[a] IC_{50} (nM)	L1210/L-PAM[b] IC_{50} (nM)	ratio[c]
brostallicin	2.13	0.64	3.3
PNU-151807	3.45	1.05	3.3
L-PAM	1097.64	5393.18	0.2
cisplatin	89.91	183.15	0.5
doxorubicin	37.84	39.56	0.9
TAM	55.30	48.90	1.1

[a] Drug cytotoxicity was determined after 48 h of continuous exposure against L1210 and L1210/L-PAM cells by counting surviving cells; IC_{50} = 50% inhibitory concentration as the mean from dose−response curves of at least six replicates. [b] Cells resistant to melphalan (L-PAM). The level of GSH in L1210 and L1210/L-PAM cells is 7.7 and 25.8 nmol/10^6cells, respectively. [c] Ratio between IC_{50} values on L1210 and L1210/L-PAM cells.

More recently it has been shown by Taq polymerase stop assay that brostallicin, in the presence of GSH/GST system, binds to a sequence different from those previously reported for TAM.[34] The possible role of GSH in the brostallicin mechanism was reinforced by in vitro and in vivo experimental data. In contrast to classical alkylating agents which are less effective in tumor cells with high GSH/GST levels, the cytotoxic activity of brostallicin and PNU-151807 was higher in L1210/L-PAM cells characterized by a 3-fold increase of GSH level in respect to the wild-type L1210 cell line.[42] Table 8 compares the in vitro cytotoxicity of L-PAM, cisplatin, TAM and doxorubicin to that of brostallicin and PNU-151807 on L1210 and L1210/L-PAM cells.

Brostallicin and PNU-151807 show more than three times higher cytotoxic activity on L1210/L-PAM cells than on the parental one. TAM and doxorubicin maintain the same cytotoxicity in both cell lines while L-PAM and cisplatin show a decreased cytotoxicity on L1210/L-PAM cells. The role of GSH on brostallicin cytotoxicity was further investigated by depleting the GSH synthesis with buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthase.[35] Both cytotoxic and apoptotic effects of brostallicin on A2780 human ovarian carcinoma cells were decreased significantly when cells were treated with BSO. Among the GST isoenzymes the π class is the stronger activator of brostallicin.[40] GSH and GSTs levels are increased and are differently expressed in several cancer types,[38,43] moreover overexpression of the GST-π isoform has been associated with carcinogenesis and the development of different tumors.[44] In further experiments, the human GST-π cDNA was transfected into A2780 human ovarian carcinoma cells and four clones of A2780, with different expression levels of GST-π, were generated. A 2- and 3-fold increase in GST-π levels resulted in a 2- and 3-fold increase in cytotoxic activity of brostallicin (Table 9). Similar results were obtained on GST-π-transfected human breast carcinoma cells (MCF-7). Brostallicin showed a 5.8 times increased cytotoxicity on GST-π-transfected versus empty vector-transfected MCF-7 cells with low GST-π expression. To complete this experiment with in vivo data, A2780 clones were implanted into

Table 9. In Vitro and in Vivo Activity of Brostallicin on Cells with Different GST Intracellular Content

cell line	GST level[a] (nmol/min/mg)	in vitro cytotoxicity[b] IC_{50} (nM)	in vivo antitumor activity[c] %TI
A2780/GST clone 8	30.7	66.9	81
A2780/GST clone 7	25.0	83.3	77
A2780/clone16	13.4	202.5	36
A2780/clone 2	11.2	>260	n.d.
MCF-7/GST	77.9	103.3	n.d.
MCF-7 neo	3.3	561.1	n.d.

[a] GST total enzymatic activity[45] (nmol/min/mg) determined using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Reaction was performed using cytosolic extracts and measuring the conversion of CDNB. The data are normalized for the amount of proteins present in each sample (spectrophotometric assay). [b] Activity in isogenic systems differing in the expression of GST-π isoenzyme. Cells were incubated with the compound for 1 h. IC_{50} = 50% inhibitory concentration as the mean from dose−response curves of at least two experiments [c] For in vivo studies A2780 human ovarian carcinoma cells were injected subcutaneously. Intravenous treatment starting on day 14 after tumor implantation q7d×2. %TI = tumor growth inhibition in treated mice with respect to control mice; n.d. = not determined.

nude mice. In the GST-π overexpressing tumors the antitumor activity of brostallicin was higher without increased toxicity.

These findings suggest that GSH/GST levels/expression affect the antitumor activity of brostallicin, with a potential value in cancer treatment, and further support the hypothesis concerning the ability of brostallicin to react with GSH, giving a reactive compound able to bind DNA.

Conclusions

In conclusion, these studies led to the identification of the α-bromoacrylamido DST derivatives as a new class of cytotoxic minor groove binders characterized by potent antitumor activity and by reduced myelotoxicity. Compounds of this class interact the DNA, but at variance with other MGB, appear unreactive in classical in vitro DNA alkylation assays. There is evidence, however, that these compounds may be activated in vivo by GSH, leading to irreversible DNA interaction. Brostallicin (PNU-166196) was selected as a lead of this new class of cytotoxic compounds and is now in Phase II clinical trials. Brostallicin shows potent antitumor activity and a very favorable cytotoxicity/myelotoxicity ratio and is well tolerated in patients (Phase I data[31]), and its antitumor activity is directly related, at least in preclinical models, to the GSH/GST levels. Considering that human tumors often show increased GST/GSH content compared to normal tissues, the compound potentially offers the unique advantage of having a specific role for the treatment of tumors characterized by constitutive or therapy-induced overexpression of GSH/GST.

Experimental Section

Chemical Materials and Methods. Reaction courses and product mixtures were routinely monitored by thin-layer chromatography on silica gel (precoated F_{254} Merk plates), and the spots were examined with UV light and visualized with aqueous $KMnO_4$. ^{1}HNMR spectra were recorded in the given solvent with a Varian 200 or 400 spectrometer. Chemical shifts are reported as (δ) values in parts per million. The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (double doublet), t (triplet), br (broad), and m (multiplet). All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. FAB mass spectra analysis were recorded on a TSQ-700 Finningan spectrometer in the positive or in the negative mode as indicated. ESI mass spectra analyses were recorded on an ion trap mass LCQ-Deca Finningan spectrometer. Elemental analyses, was performed using EA 1110 CHNS−O C. E. Instruments Elemental Analyzer and were within 0.4% of the theorical values calculated for C, H, Br, Cl, and N. Column chromatography was carried out Merck silica gel (230−240 mash). All compounds obtained commercially were used without further purification. In high-pressure hydrogenation experiments, a Parr shaker on a high-pressure autoclave was used.

Method A. General Procedure for the Synthesis of Compounds 3−26. A solution of the desired acid (6 mmol) and thionyl chloride (20 mmol) in 10 mL of dry toluene was refluxed for 3 h. The solvent was allowed under vacuum, the residue was dissolved in dry dioxane (10 mL) and added dropwise to a solution of the appropriate amine (3 mmol) and $NaHCO_3$ (12 mmol) in water (10 mL). The reaction was stirred at room temperature for 2 h and acidified with 1 N HCl until pH = 3, the solvent allowed under vacuum, and the residue purified by flash chromatography on silica gel using a DCM/MeOH mixture as eluent.

Method B. General Procedure for the Synthesis of Compounds 3−26. A solution of the appropriate acid (6 mmol) in dry THF (15 mL) was cooled in an ice bath, and DCC (3 mmol) was added. The reaction was stirred at room temperature for 30 min, the DCU was removed by filtration, and the organic solution was added dropwise to a solution of the selected amine (3 mmol) and $NaHCO_3$ (6 mmol) in water (10 mL). The reaction was stirred overnight, the solution was acidified with 1 N HCl until pH = 3, the solvent was evaporated under vacuum, and the residue was purified by flash chromatography on silica gel using a DCM/MeOH mixture as eluant.

***N*-(5-{[(5-{[(5-{[(3-Amino-3-iminopropyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1-*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*pyrrol-3-yl)-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (3).** Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)-amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (546 mg, 2 mmol), and 4-amino-*N*-(5-{[(5-{[(2-{[amino(imino)methyl]amino}-ethyl)amino]carbonyl}1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*pyrrol-3-yl)-1-methyl-*H*-pyrrole-2-carboxamide dihydrochloride **36** (526 mg, 1 mmol), after flash chromatography (8:2 DCM/MeOH), compound **3** was obtained as a pale yellow solid (447 mg, 60% yield): ^{1}H NMR (DMSO-d_6): δ 2.81 (t, J = 6.5 Hz, 2H), 3.48 (m, 2H), 3.88 (s, 3H), 4.00 (s, 3H), 4.01 (s, 3H), 4.02 (s, 3H), 6.22 (d, J = 2.8 Hz, 1H), 6.67 (d, J = 2.8 Hz, 1H), 6.92 (d, J = 1.8 Hz, 1H), 7.03 (m, 3H), 7.18 (d, J = 1.8 Hz, 1H), 7.22 (m, 3H), 8.22 (t, J = 5.8 Hz, 1H), 8.62 (bs, 2H), 8.99 (bs, 2H), 9.91 (s, 1H), 9.95 (s, 1H), 9,98 (s, 1H), 10.36 (s, 1H); MS (FAB) m/z 709 [(M + H)$^+$]; Anal. ($C_{30}H_{34}BrN_{11}O_5$·HCl) C, H, N.

***N*-(5-{[(5-{[(5-{[(3-Amino-3-iminopropyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl1*H*-pyrrol-3-yl)-4-[(2-chloroacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (4).** Following the general procedure of method B, starting from 4-[(2-chloroacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **32** (456 mg, 2 mmol) and 4-amino-*N*-(5-{[(5-{[(2-{[amino(imino)methyl]amino}ethyl)-amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **36** (526 mg, 1 mmol), after flash chromatography (8:2 DCM/MeOH), compound **4** was obtained as a pale yellow solid (210 mg, 30% yield): ^{1}H NMR (DMSO-d_6): δ 2.62 (t, J = 6.5 Hz, 2H), 3.48 (m, 2H), 3.87 (s, 3H), 3.89 (s, 3H), 4.00 (s, 3H), 4.02 (s, 3H), 5.99 (d, J = 2.8 Hz, 1H), 6.39 (d, J = 2.8 Hz, 1H), 6.90−7.3 (m, 8H), 8.20 (t, J = 5.8 Hz, 1H), 8.80 (bs, 2H), 9.00 (bs, 2H), 9.90 (s, 2H), 9.93 (s, 1H), 10.30 (s, 1H); MS (FAB) m/z 664 [(M + H)$^+$]; Anal. ($C_{30}H_{34}ClN_{11}O_5$·HCl) C, H, N.

***N*-(5-{[(5-{[(5-{[(3-Amino-3-iminopropyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-fluoroacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (5).** Following the general procedure of method A, starting from 4-[(2-fluoroacryloyl)-amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **33** (424 mg, 2 mmol) and 4-amino-*N*-(5-{[(5-{[(2-{[amino(imino)methyl]amino}-ethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **36** (526 mg, 1 mmol), after flash chromatography (8:2 DCM/MeOH), compound **5** was obtained as a pale yellow solid (424 mg, 62% yield): ^{1}H NMR (DMSO-d_6): δ 2.61 (t, J = 6.5 Hz, 2H), 3.51 (m, 2H), 3.85 (s, 3H), 3.87 (s, 3H), 3.88 (s, 3H), 4.01 (s, 3H), 5.37 (dd, J = 3.5, 15.8 Hz, 1H), 5.63 (dd, J = 3.5, 47.9 Hz, 1H), 6.95 (d, J = 1.7 Hz, 1H), 7.07 (m, 3H), 7.19 (d, J = 1.7 Hz, 1H), 7.25 (m, 3H), 8.23 (t, J = 5.8 Hz, 1H), 8.77 (bs, 4H), 9.93 (s, 1H), 9.96 (s, 1H), 10.01 (s, 1H), 10.51 (s, 1H); MS (FAB) m/z 648 [(M + H)$^+$]; Anal. (C_{30} $H_{34}FN_{11}O_5$·HCl) C, H, N.

4-(Acryloylamino)-*N*-(5-{[(5-{[(3-amino-3-iminopropyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]-carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (6). Following the general procedure of method A, starting from 4-acrylamino-1-methyl-1*H*-pyrrole-2-carboxylic acid **34** (388 mg, 2 mmol) and 4-amino-*N*-(5-{[(5-{[(2-{[amino(imino)methyl]amino}ethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **36** (526 mg, 1 mmol), after flash chromatography (8:2 DCM/MeOH), compound **6** was obtained as a ivory solid (466 mg, 70% yield): ^{1}H NMR (DMSO-d_6): δ 2.62 (m, 2H), 3.52 (m, 2H), 3.79 (s, 3H), 3.81 (s, 3H), 3.86 (s, 3H), 3.89 (s, 3H), 5.67 (dd, J = 5.0, 1.2 Hz, 1H), 6.18 (dd, J = 8.5, 1.2 Hz, 1H), 6.41 (dd, J = 8.5, 5.0 Hz, 1H), 6.92 (m, 2H), 7.09 (m, 2H), 7.18 (d, J = 1.8 Hz, 1H), 7.22 (m, 2H), 7.23(d, J = 1.8 Hz, 1H), 8.22 (m, 1H), 8.50−9.00 (bs, 4H), 9.91 (s, 1H), 9.95 (s, 1H), 9,98 (s, 1H), 10.22 (s, 1H); MS (FAB) m/z 630 [(M + H)$^+$]; Anal. ($C_{30}H_{35}N_{11}O_5$·HCl) C, H, N.

***N*-(5-{[(5-{[(5-{[(5-{[(3-Amino-3-iminopropyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (7).** Following the general procedure of method A, starting from 4-[({4-[(2-bromoacryloyl)-amino]-1-methyl-1*H*-pyrrol-2-yl}carbonyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **35** (790 mg, 2 mmol) and 4-amino-*N*-(5-{[(5-{[(2-{[amino(imino)methyl]amino}ethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **36** (526 mg, 1 mmol), after flash chromatography (8:2 DCM/MeOH), compound **7** was obtained as an ivory solid (503 mg, 58% yield): ^{1}H NMR-(DMSO-d_6): δ 2.61 (t, J = 6.5 Hz, 2H), 3.48 (m, 2H), 3.80(s, 3H), 3.83(s, 6H), 3.84 (s, 3H), 3.85 (s, 3H), 6.21 (d, J = 2.8 Hz, 1H), 6.79 (d, J = 2.8 Hz, 1H), 6.94 (d, J = 1.7 Hz, 1H), 7.05 (m, 4H), 7.17 (d, J = 1.7 Hz, 1H), 7.22 (m, 4H), 8.19 (t, J = 5.8 Hz, 1H), 8.63 (bs, 2H), 8.91 (bs, 2H), 9.90 (s, 1H), 9.93 (bs, 2H), 9.96 (s, 1H), 10.30 (s, 1H); MS (FAB) m/z 831 [(M + H)$^+$]; Anal. ($C_{36}H_{40}BrN_{13}O_6$·HCl) C, H, N.

***N*-(5-{[(5-{[(3-Amino-3-iminopropyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (8).** Following the general procedure of method B, starting from 2-bromoacrylic acid **28** (453 mg, 3 mmol) and 4-amino-*N*-(5-{[(5-{[(2-{[amino-(imino)methyl]amino}ethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **36** (789 mg, 1.5 mmol), after flash chromatography (8:2 DCM/MeOH), compound **8** was obtained as a yellow solid (249 mg, 40% yield): ^{1}H NMR (DMSO-d_6): δ 2.59 (t, J = 6.5 Hz, 2H), 3.46 (m, 2H), 3.78 (s, 3H), 3.79 (s, 3H), 3.81 (s, 3H), 6.20 (d, J = 2.8 Hz, 1H), 6.64 (d, J = 2.8 Hz, 1H), 6.91 (d, J = 1.8 Hz, 1H), 7.03 (m, 2H), 7.18 (d, J = 1.8 Hz, 1H), 7.21 (m, 2H), 8.20 (t, J = 5.8 Hz, 1H), 8.56 (bs, 2H), 8.92 (bs, 2H), 9.89 (s, 1H), 9.93 (s, 1H), 10.31 (s, 1H); MS (FAB) m/z 587 [(M + H)$^+$]; Anal. ($C_{24}H_{28}$-BrN_9O_4·HCl) C, H, N.

***N*-(5-{[(3-Amino-3-iminopropyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (9).** Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **32** (546 mg, 2 mmol), and 4-amino-*N*-(3-amino-3-imino-propyl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **52** (282 mg, 1 mmol), after flash chromatography (8:2 DCM/MeOH), compound **9** was obtained as a yellow solid (200 mg, 40% yield): ^{1}H NMR (DMSO-d_6): δ 2.60 (t, J = 6.5 Hz, 2H), 3.48 (m, 2H), 3.81 (s, 3H), 3.84 (s, 3H), 6.22 (d, J = 2.8 Hz, 1H), 6.71 (d, J = 2.8 Hz, 1H), 6.94 (d, J = 1.8 Hz, 1H), 7.04 (d, J = 1.8 Hz, 1H), 7.20 (d, J = 1.8 Hz, 1H), 7.22 (d, J = 1.8 Hz, 1H), 8.22 (t, J = 5.8 Hz, 1H), 8.62 (bs, 2H), 9.02 (bs, 2H), 9.98 (s, 1H), 10.38 (s, 1H); MS (ESI) m/z 465 [(M + H)$^+$]; Anal. ($C_{18}H_{22}BrN_7O_3$·HCl) C, H, N.

***N*-(3-Amino-3-iminopropyl)-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (10).** Following the general procedure of method B, starting from 2-bromoacrylic acid **28** (151 mg, 1 mmol), and 4-amino-*N*-(3-amino-3-iminopropyl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **52** (141 mg, 0.5 mmol), after flash chromatography (8:2 DCM/MeOH), compound **10** was obtained as a ivory solid (95 mg, 50% yield): ^{1}H NMR (DMSO-d_6): δ 2.59 (t, J = 6.5 Hz, 2H), 3.46 (m, 2H), 3.78 (s, 3H), 6.19 (d, J = 2.8 Hz, 1H), 6.66 (d, J = 2.8 Hz, 1H), 6.91 (d, J = 1.8 Hz, 1H), 7.18 (d, J = 1.8 Hz, 1H), 8.22 (t, J = 5.8 Hz, 1H), 8.58 (bs, 2H), 8.92 (bs, 2H), 10.24 (s, 1H); MS (ESI) m/z 343 [(M + H)$^+$]; Anal. ($C_{12}H_{16}BrN_5O_2$·HCl) C, H, N.

***N*-(5-{[(5-{[(3-Amino-3-iminopropyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-chloroacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (11).** Following the general procedure of method B, starting from 2-chloroacrylic acid **29** (212 mg, 2 mmol), and 4-amino-*N*-(5-{[(5-{[(2-{[amino-(imino)methyl]amino}ethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **36** (526 mg, 1 mmol), after flash chromatography (8:2 DCM/MeOH), compound **11** was obtained as an hazel solid (346 mg, 60% yield): ^{1}H NMR (DMSO-d_6): δ 2.65 (t, J = 6.5 Hz, 2H), 3.50 (m, 2H), 3.80 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 5.98 (d, J = 2.8 Hz, 1H), 6.40 (d, J = 2.8 Hz, 1H), 6.90−7.30 (m, 6H), 8.20 (t, J = 5.8 Hz, 1H), 8.75 (bs, 2H), 9.04 (bs, 2H), 9.89 (s, 1H), 9.95 (s, 1H), 10.32 (s, 1H); MS (FAB) m/z 542 [(M + H)$^+$]; Anal. ($C_{24}H_{28}$-ClN_9O_4·HCl) C, H, N.

***N*-(5-{[(5-{[(3-Amino-3-iminopropyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-fluoroacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (12).** Following the general procedure of method A, starting from 2-fluoroacrylic acid **30** (180 mg, 2 mmol) and 4-amino-*N*-(5-{[(5-{[(2-{[amino-(imino)methyl]amino}ethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **36** (526 mg, 1 mmol), after flash chromatography (8:2 DCM/MeOH), compound **12** was obtained as an hazel solid (281 mg, 50% yield): ^{1}H NMR (DMSO-d_6): δ 2.59 (t, J = 6.5 Hz, 2H), 3.44 (m, 2H), 3.78 (s, 3H), 3.79 (s, 3H), 3.81 (s, 3H), 5.29 (dd, J = 3.5, 15.8 Hz, 1H), 5.49 (dd, J = 3.5, 47.9 Hz, 1H), 6.91 (d, J = 1.8 Hz, 1H), 7.01 (d, J = 1.8 Hz, 1H), 7.03 (d, J = 1.8 Hz, 1H), 7.17 (d, J = 1.8 Hz, 1H), 7.19 (d, J = 1.8 Hz, 1H), 7.21 (d, J = 1.8 Hz, 1H), 8.19 (t, J = 5.8 Hz, 1H), 8.50 (bs, 2H), 8.92 (bs, 2H), 9.89 (s, 1H), 9.93 (s, 1H), 10.42 (s, 1H); MS (FAB) m/z 526 [(M + H)$^+$]; Anal. ($C_{24}H_{28}FN_9O_4$·HCl) C, H, N.

***N*-[5-({[5-({[5-({[3-Amino-3-(methylimino)propyl]amino}-carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-**

2-carboxamide Hydrochloride (13). Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)-amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (546 mg, 2.0 mmol) and 4-amino-*N*-[5-({[5-({[3-amino-3-(methylimino)-propyl]amino}carbonyl)-1-methyl-1*H*-yrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **37** (540 mg, 1.0 mmol), after flash chromatography (8:2 DCM/MeOH), compound **13** was obtained as an yellow solid (493 mg, 65% yield): ^{1}H NMR (DMSO-d_6): δ 2.59 (m, 2H), 2.79 (s, 3H), 3.48 (m, 2H), 3.80 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 3.86 (s, 3H), 6.22 (d, J = 2.8 Hz, 1H), 6.68 (d, J = 2.8 Hz, 1H), 6.90 (d, J = 1.8 Hz, 1H), 7.02 (m, 3H), 7.15 (d, J = 1.8 Hz, 1H), 7.20 (m, 3H), 8.20 (t, J = 5.9 Hz, 1H), 8.50 (bs, 1H), 9.10 (bs, 1H), 9.50 (bs, 1H), 9.92 (s, 1H), 9.95 (s, 1H), 9.98 (s, 1H), 10.32 (s, 1H); MS (FAB) *m/z* 723 [(M + H)$^+$]; Anal. ($C_{31}H_{36}BrN_{11}O_5$·HCl) C, H, N.

4-[(2-Bromoacryloyl)amino]-1-methyl-*N*-[1-methyl-5-({[1-methyl-5-({[1-methyl-5-({[3-(methylamino)-3-(methylimino)propyl]amino}carbonyl)-1*H*-pyrrol-3-yl]-amino}carbonyl)-1*H*-pyrrol-3-yl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide Hydrochloride (14). Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (200 mg, 0.73 mmol) and 4-amino-1-methyl-*N*-[1-methyl-5-({[1-methyl-5-({[3-(methylamino)-3-(methylimino)-propyl]amino}carbonyl)-1*H*-pyrrol-3-yl]amino}carbonyl)-1*H*-pyrrol-3-yl]-1*H*-pyrrole-2-carboxamide dihydrochloride **41** (250 mg, 0.45 mmol), after flash chromatography (8.5:1.5 DCM/MeOH), compound **14** was obtained as white solid (185 mg, 53% yield): ^{1}H NMR (DMSO-d_6): δ 2.73 (t, J = 6.5 Hz, 2H), 2.79 (s, 3H), 3.00 (s, 3H), 3.42 (m, 2H), 3.80 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 3.86 (s, 6H), 6.19 (d, J = 2.9 Hz, 1H), 6.71 (d, J = 2.9 Hz, 1H), 6.90 (d, J = 1.8 Hz, 1H), 7.02 (m, 3H), 7.18 (d, J = 1.8 Hz, 1H), 7.21 (m, 3H), 8.25 (t, J = 6.0 Hz, 1H), 9.20 (bs, 2H), 9.94 (s, 1H), 9.96 (s, 1H), 9.98 (s, 1H), 10.34 (s, 1H); MS (ESI) *m/z* 737 [(M + H)$^+$]; Anal. ($C_{32}H_{38}BrN_{11}O_5$·HCl) C, H, N.

4-[(2-Bromoacryloyl)amino]-*N*-[5-({[5-({[5-({[3-(dimethylamino)-3-iminopropyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (15). Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (142 mg, 0.52 mmol) and 4-amino-*N*-[5-({[5-({[3-(dimethylamino)-3-iminopropyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}-carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **38** (140 mg, 0.26 mmol), after flash chromatography (8.5:1.5 DCM/MeOH), compound **15** was obtained as pale yellow solid (96 mg, 48% yield): ^{1}H NMR (DMSO-d_6): δ 2.77 (t, J = 6.5 Hz, 2H), 3.03 (bs, 3H), 3.22 (s, 3H), 3.46 (m, 2H), 3.80 (s, 3H), 3.83 (s, 3H), 3.84 (s, 6H), 6.22 (d, J = 2.9 Hz, 1H), 6.70 (d, J = 2.9 Hz, 1H), 6.92 (d, J = 1.8 Hz, 1H), 7.06 (m, 3H), 7.20 (d, J = 1.8 Hz, 1H), 7.22 (m, 3H), 8.30 (t, J = 6.0 Hz, 1H), 9.00 (bs, 2H), 9.94 (s, 1H), 9.95 (s, 1H), 9.99 (s, 1H), 10.37 (s, 1H); MS (ESI) *m/z* 737 [(M + H)$^+$]; Anal. ($C_{32}H_{38}BrN_{11}O_5$·HCl) C, H, N.

4-[(2-Bromoacryloyl)amino]-*N*-[5-({[5-({[5-({[2-(4,5-dihydro-1*H*-imidazol-2-yl)ethyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (16). Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (164 mg, 0.6 mmol) and 4-amino-*N*-[5-({[5-({[2-(4,5-dihydro-1*H*-imidazol-2-yl)ethyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **42** (165 mg, 0.3 mmol), after flash chromatography (8.5:1.5 DCM/MeOH), compound **16** was obtained as pale yellow solid (184 mg, 80% yield): ^{1}H NMR (DMSO-d_6): δ 2.68 (t, J = 6.5 Hz, 2H), 3.42 (m, 2H), 3.78 (s, 3H), 3.80 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 6.22 (d, J = 2.8 Hz, 1H), 6.71 (d, J = 2.8 Hz, 1H), 6.91(d, J = 1.8 Hz, 1H), 7.08 (m, 3H), 7.18 (d, J = 1.8 Hz, 1H), 7.22 (m, 3H), 8.25 (t, J = 5.9 Hz, 1H), 9.91 (s, 1H), 9.94 (s, 1H), 9.95 (s, 1H), 10.00 (bs, 2H) 10.38 (s, 1H); MS (ESI) *m/z* 735 (M + H)$^+$]; Anal. ($C_{32}H_{36}BrN_{11}O_5$·HCl) C, H, N.

***N*-(5-{[(5-{[(5-{[(2-{[Amino(imino)methyl]amino}ethyl)-amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (17).** Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (840 mg, 3.1 mmol) and 4-amino-*N*-(5-{[(5-{[(2-{[amino(imino)methyl]-amino}ethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]-carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide **45** (866 mg, 1.6 mmol), after flash chromatography (8:2 DCM/MeOH), compound **17** was obtained as an ivory solid (730 mg, 60% yield): ^{1}H NMR (DMSO-d_6): δ 3.30 (m, 4H), 3.80 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 6.21 (d, J = 2.9 Hz, 1H), 6.68 (d, J = 2.9 Hz, 1H), 6.91 (d, J = 1.8 Hz, 1H), 7.04 (m, 3H), 7.19 (d, J = 1.8 Hz, 1H), 7.2 (bs, 4H), 7.22 (m, 3H), 7.56 (t, J = 5.9 Hz, 1H), 8.10 (t, J = 5.9 Hz, 1H), 9.90 (s, 1H), 9.92 (s, 1H), 9.95 (s, 1H), 10.30 (s, 1H); MS (FAB) *m/z* 724 [(M + H)$^+$]; Anal. ($C_{30}H_{35}BrN_{12}O_5$·HCl) C, H, N.

***N*-(5-{[(5-{[(5-{[(2-{[Amino(imino)methyl]amino}ethyl)-amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-fluoroacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (18).** Following the general procedure of method A, starting from 4-[(2-fluoroacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **30** (339 mg, 1.6 mmol) and 4-amino-*N*-(5-{[(5-{[(2-{[amino(imino)methyl]-amino}ethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]-carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide **45** (433 mg, 0.8 mmol), after flash chromatography (8:2 DCM/MeOH), compound **18** was obtained as an pale yellow solid (329 mg, 60% yield): ^{1}H NMR (DMSO-d_6): δ 3.30 (m, 4H), 3.81 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 5.35 (dd, J = 3.5, 15.8 Hz), 5.61 (dd, J = 3.5, 47.9 Hz), 6.93 (d, J = 1.8 Hz, 1H), 7.03 (m, 3H), 7.19 (d, J = 1.8 Hz, 1H), 7.21 (bs, 4H), 7.22 (m, 2H), 7.23 (d, J = 1.8 Hz, 1H), 7.56 (t, J = 5.9 Hz, 1H), 8.18 (t, J = 5.9 Hz, 1H), 9.90 (s, 1H), 9.92 (s, 1H), 9.98 (s, 1H), 10.20 (s, 1H); MS (FAB) *m/z* 663 [(M + H)$^+$]; Anal. ($C_{30}H_{35}FN_{12}O_5$·HCl) C, H, N.

4-[(2-Bromoacryloyl)amino]-1-methyl-*N*-(1-methyl-5-{[(1-methyl-5-{[(1-methyl-5-{[(2-{[(methylamino)-(methylimino)methyl]amino}ethyl)amino]carbonyl}-1*H*-pyrrol-3-yl)amino]carbonyl}-1*H*-pyrrol-3-yl)amino]-carbonyl}-1*H*-pyrrol-3-yl)-1*H*-pyrrole-2-carboxamide Hydrochloride (19). Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (273 mg, 1 mmol) and 4-amino-1-methyl-*N*-(1-methyl-5-{[(1-methyl-5-{[(2-{[(methylamino)-(methylimino)methyl]amino}ethyl)amino]carbonyl}-1*H*-pyrrol-3-yl)amino]carbonyl}-1*H*-pyrrol-3-yl)-1*H*-pyrrole-2-carboxamide dihydrochloride **48** (285 mg, 0.5 mmol), after flash chromatography (8:2 DCM/MeOH), compound **19** was obtained as an ivory solid (197 mg, 50% yield): ^{1}H NMR (DMSO-d_6): δ 2.74 (s, 3H), 2.75 (s, 3H), 3.37 (m, 4H), 3.79 (s, 3H), 3.81 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 6.21 (d, J = 2.9 Hz, 1H), 6.66 (d, J = 2.9 Hz, 1H), 6.92 (d, J = 1.8 Hz, 1H), 7.03 (m, 3H), 7.19 (d, J = 1.8 Hz, 1H), 7.21 (m, 3H), 7.42 (m, 1H), 7.55 (bs, 2H), 8.18 (t, J = 5.9 Hz, 1H), 9.89 (s, 1H), 9.90 (s, 1H), 9.94 (s, 1H), 10.30 (s, 1H); MS (ESI) *m/z* 752 [(M + H)$^+$]; Anal. ($C_{32}H_{39}$-$BrN_{12}O_5$·HCl) C, H, N.

***N*-(5-{[(5-{[(5-{[(3-{[Amino(imino)methyl]amino}propyl)-amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (20).** Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (273 mg, 1 mmol) and 4-amino-*N*-(5-{[(5-{[(3-{[amino(imino)methyl]-amino}propyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **46** (277 mg, 0.5 mmol), after flash

chromatography (8:2 DCM/MeOH), compound **20** was obtained as a pale yellow solid (194 mg, 50% yield): ^{1}H NMR (DMSO-d_6): δ 1.68 (m, 2H), 3.18 (m, 2H), 3.21 (m, 2H), 3.78 (s, 3H), 3.81 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 6.21 (d, J = 2.9 Hz, 1H), 6.64 (d, J = 2.9 Hz, 1H), 6.70−7.40 (m, 4H), 6.92 (d, J = 1.8 Hz, 1H), 7.03 (m, 3H), 7.18 (d, J = 1.8 Hz, 1H), 7.21 (m, 3H), 7.46 (m, 1H), 8.02 (t, J = 5.9 Hz, 1H), 9.84 (s, 1H), 9.90 (s, 1H), 9.92 (s, 1H), 10.28 (s, 1H); MS (ESI) m/z 738 [(M + H)$^+$]; Anal. ($C_{31}H_{37}BrN_{12}O_5$·HCl) C, H, N.

***N*-(5-{[(5-{[(5-{[(4-{[Amino(imino)methyl]amino}butyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (21).** Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (273 mg, 1 mmol) and 4-amino-*N*-(5-{[(5-{[(4-{[amino(imino)methyl]amino}butyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **47** (285 mg, 0.5 mmol), after flash chromatography (8:2 DCM/MeOH), compound **21** was obtained as a white solid (213 mg, 54% yield): ^{1}H NMR (DMSO-d_6): δ 1.48 (m, 4H), 3.12 (m, 2H), 3.18 (m, 2H), 3.78 (s, 3H), 3.81 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 6.20 (d, J = 2.9 Hz, 1H), 6.66 (d, J = 2.9 Hz, 1H), 6.88 (d, J = 1.8 Hz, 1H), 7.04 (m, 3H), 7.14 (d, J = 1.8 Hz, 1H), 7.22 (m, 3H), 6.60−7.40 (bs, 4H), 7.51 (t, J = 5.9 Hz, 1H), 8.02 (t, J = 5.9 Hz, 1H), 9.85 (s, 1H), 9.91 (s, 1H), 9.94 (s, 1H), 10.28 (s, 1H); MS (ESI) m/z 752 [(M + H)$^+$]; Anal. ($C_{32}H_{39}BrN_{12}O_5$·HCl) C, H, N.

4-[(2-Bromoacryloyl)amino]-*N*-[5-({[5-({[2-(4,5-dihydro-1*H*-imidazol-2-ylamino)ethyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (22). Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (273 mg, 1 mmol) and 4-amino-*N*-[5-({[5-({[2-(4,5-dihydro-1*H*-imidazol-2-ylamino)ethyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide dihydrochloride **49** (276 mg, 0.5 mmol), after flash chromatography (8:2 DCM/MeOH; 8/2), compound **22** was obtained as a white solid (177 mg, 45% yield): ^{1}H NMR (DMSO-d_6): δ 3.26 (m, 4H), 3.59 (m, 4H), 3.79 (s, 3H), 3.81 (s, 3H), 3.82 (s, 3H), 3.83 (s, 3H), 6.21 (d, J = 2.9 Hz, 1H), 6.68 (d, J = 2.9 Hz, 1H), 6.92 (d, J = 1.8 Hz, 1H), 7.02 (m, 3H), 7.19 (d, J = 1.8 Hz, 1H), 7.22 (m, 3H), 7.40−8.60 (bs, 2H), 8.08 (m, 1H), 8.22 (m, 1H), 9.90 (s, 1H), 9.92 (s, 1H), 9.96 (s, 1H) 10.28 (s, 1H); MS (ESI) m/z 750 [(M + H)$^+$]; Anal. ($C_{32}H_{37}BrN_{12}O_5$·HCl) C, H, N.

***N*-{5-[({5-[({5-[({2-[(Aminocarbonyl)amino]ethyl}amino)carbonyl]-1-methyl-1*H*-pyrrol-3-yl}amino)carbonyl]-1-methyl-1*H*-pyrrol-3-yl}amino)carbonyl]-1-methyl-1*H*-pyrrol-3-yl}-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide (23).** Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (273 mg, 1 mmol) and 4-amino-*N*-{5-[({5-[({2-[(aminocarbonyl)amino]-ethyl}amino)carbonyl]-1-methyl-1*H*-yrrol-3-yl}amino)carbonyl]-1-methyl-1*H*-pyrrol-3-yl}-1-methyl-1*H*-pyrrole-2-carboxamide hydrochloride **50** (252 mg, 0.5 mmol), after flash chromatography (8:2 DCM/MeOH), compound **23** was obtained as a pale yellow solid (190 mg, 50% yield): ^{1}H NMR (DMSO-d_6): δ 3.10 (m, 2H), 3.18 (m, 2H), 3.79 (s, 3H), 3.83 (s, 3H), 3.84 (s, 6H), 5.48 (s, 2H), 6.06 (m, 1H), 6.21 (d, J = 2.9 Hz, 1H), 6.68 (d, J = 2.9 Hz, 1H), 6.83 (d, J = 1.8 Hz, 1H), 7.04 (m, 3H), 7.18 (d, J = 1.8 Hz, 1H), 7.21 (m, 3H), 8.02 (t, J = 5.9 Hz, 1H), 9.87 (s, 1H), 9.91 (s, 1H), 9.94 (s, 1H), 10.29 (s, 1H); MS (ESI) m/z 725 [(M + H)$^+$]; Anal. ($C_{30}H_{34}BrN_{11}O_6$) C, H, N.

***N*-[5-({[5-({[5-({[3-Amino-3-(hydroxyimino)propyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide Hydrochloride (24).** Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (546 mg, 2.0 mmol) and 4-amino-*N*-[5-({[5-({[3-amino-3-(hydroxyimino)propyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide hydrochloride **39** (506 mg, 1.0 mmol), after flash chromatography (8.5:1.5 DCM/MeOH), compound **24** was obtained as a pale hazel solid (470 mg, 65% yield): ^{1}H NMR (DMSO-d_6): δ 2.31 (m, 2H), 3.38 (m, 2H), 3.79 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 6.21 (d, J = 2.9 Hz, 1H), 6.69 (d, J = 2.9 Hz, 1H), 6.90 (d, J = 1.8 Hz, 1H), 7.02 (m, 3H), 7.18 (d, J = 1.8 Hz, 1H), 7.21 (m, 3H), 7.80−8.20 (bs, 2H), 8.12 (t, J = 5.9 Hz, 1H), 9.88 (s, 1H), 9.91 (s, 1H), 9.94 (s, 1H), 10.27 (s, 1H), 12.22 (bs, 1H); MS (ESI) m/z 725 (M + H)$^+$]; Anal. (C_{30} H_{34} Br N_{11} O_6) C, H, N.

***N*-[5-({[5-({[5-({[3-Amino-3-(cyanoimino)propyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide (25).** Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (164 mg, 0.60 mmol) and 4-amino-*N*-[5-({[5-({[3-amino-3-(cyanoimino)propyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide hydrochloride **40** (175 mg, 0.34 mmol), after flash chromatography (9:1 DCM/MeOH), compound **25** was obtained as an hazel solid (150 mg, 60% yield): ^{1}H NMR (DMSO-d_6): δ 2.60 (bs, 2H), 3.45 (bs, 2H), 3.79 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 3.86 (s, 3H), 6.22 (d, J = 2.9 Hz, 1H), 6.67 (d, J = 2.9 Hz, 1H), 6.90 (d, J = 1.8 Hz, 1H), 7.04 (m, 3H), 7.19 (d, J = 1.8 Hz, 1H), 7.22 (m, 2H), 8.08 (bs, 1H), 8.30 (bs, 2H), 9.88 (s, 1H), 9.92 (s, 1H), 9.95 (s, 1H), 10.27 (s, 1H); MS (FAB) m/z 732 [(M − H)$^-$]; Anal. ($C_{31}H_{33}BrN_{12}O_5$) C, H, N.

***N*-{5-[({5-[({5-[({2-(Carboxamide)ethyl}amino)carbonyl]-1-methyl-1*H*-pyrrol-3-yl}amino)carbonyl]-1-methyl-1*H*-pyrrol-3-yl}amino)carbonyl]-1-methyl-1*H*-pyrrol-3-yl}-4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxamide (26).** Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (163 mg, 0.60 mmol) and 4-amino-*N*-[5-({[5-({[3-amino-3-(cyanoimino)propyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-1-methyl-1*H*-pyrrole-2-carboxamide hydrochloride **44** (175 mg, 0.34 mmol), after flash chromatography (9:1 DCM/MeOH), compound **26** was obtained as a yellow solid (145 mg, 60% yield): ^{1}H NMR (DMSO-d_6): δ 2.28 (t, J = 6.5 Hz, 2H), 3.34 (m, 2H), 3.79 (s, 3H), 3.81 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 6.21 (d, J = 2.9 Hz, 1H), 6.66 (d, J = 2.9 Hz, 1H), 6.79 (bs, 1H), 6.82 (d, J = 1.8 Hz, 1H), 7.02 (m, 3H), 7.18 (d, J = 1.8 Hz, 1H), 7.21 (m, 3H), 7.31 (bs, 1H), 7.98 (t, J = 5.8 Hz, 1H), 9.86 (s, 1H), 9.91 (s, 1H), 9.96 (s, 1H), 10.28 (s, 1H); MS (FAB) m/z 710 [(M + H)$^+$]; Anal. ($C_{30}H_{33}BrN_{10}O_6$) C, H, N.

4-[(2-Bromoacryloyl)amino]-*N*-(5-{[(5-{[(5-{[(2-cyanoethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide (27). Following the general procedure of method A, starting from 4-[(2-bromoacryloyl)amino]-1-methyl-1*H*-pyrrole-2-carboxylic acid **31** (163 mg, 0.60 mmol), and 4-amino-*N*-(5-{[(5-{[(2-cyanoethyl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)amino]carbonyl}-1-methyl-1*H*-pyrrol-3-yl)-1-methyl-1*H*-pyrrole-2-carboxamide hydrochloride **43** (142 mg, 0.30 mmol), after flash chromatography (9.5:0.5 DCM/MeOH), compound **27** was obtained as an ivory solid (125 mg, 60% yield): ^{1}H NMR (DMSO-d_6): δ 2.75 (t, J = 6.5 Hz, 2H), 3.42 (m, 2H), 3.60 (s, 3H), 3.87 (s, 3H), 3.88 (s, 6H), 6.25 (d, J = 2.9 Hz, 1H), 6.70 (d, J = 2.9 Hz, 1H), 6.92 (d, J = 1.8 Hz, 1H), 7.03 (m, 3H), 7.15 (m, 4H), 8.36 (t, J = 5.9 Hz, 1H), 9.95 (s, 1H), 9.97 (s, 1H), 10.00 (s, 1H), 10.32 (s, 1H); MS (FAB) m/z 692 [(M + H)$^+$]; Anal. ($C_{30}H_{31}BrN_{10}O_5$) C, H, N.

Tumor Models. L1210 murine lymphocytic leukemia was from NCI (Frederick, MD). Human lung (N592 and A549), colon (HCT-116 and HT-29), and prostatic (DU 145) carcinomas were from American Type Culture Collection (Rockville,

MD). Human pancreatic (CAPAN 1) and renal (Caki-2) carcinomas were distributed by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy). Human ovarian (A2780 and H-207) and gastric (GTL 16) carcinomas were provided by Dr. Ozols, NCI (Frederick, MD), Dr. G. Pezzoni (Milan, Italy), and Prof. P. Comoglio, University of Turin (Turin, Italy), respectively.

In Vitro Growth Inhibition Assay. Concentration−response curves were generated to determine the concentration required for 50% inhibition of growth (IC_{50}). The murine leukemia L1210 and the subline resistant to L-PAM (L1210/L-PAM) were grown in RPMI-1640 (Gibco) supplemented with 10% FCS. Exponentially growing cells were seeded and exposed to various concentrations of drug immediately after seeding. The antiproliferative activity of the drug was evaluated after different times of treatment as reported in the tables. A2780 clones overexpressing the human GST-π gene were obtained after calcium phosphate-mediated transfection of parental cells with the human GST-π cDNA and selection in medium containing 500 μg/mL of G418. The human breast carcinoma (MCF-7) cell line and its clone overexpressing the human GST-π gene were grown as reported by Moscow.[46] All cell lines were maintained at 37 °C, 5% CO_2 in RPMI-1640 medium supplemented with 10% FCS. Exponentially growing cells were seeded and exposed to various drug concentrations. Drug cytotoxicity against L1210 and L1210/L-PAM was evaluated by counting surviving cells on a Coulter ZM Cell Counter (Coulter Electronics, Hialeath, FL). Drug-induced cytotoxicity in A2780 and MCF-7 cells was determined using the MTT test in 96-well plates.[47]

In Vivo Activity. Animals. Two to three month old female inbred DBA/2 and BALB/c x DBA/2 (CD2F1) mice, weighing 20−22 g, were used for L1210 murine leukemia. They were kept under standard laboratory conditions. Four to six week old female nude swiss Nu/Nu and HSD:Nu/Nu mice, weighing 20−25 g, were employed in experiments with human tumors. They were maintained under specific pathogen-free conditions and provided sterile food and water at libitum. Mice were supplied by Charles River (Calco, Lecco, Italy). Animal health was routinely tested for the absence of antibodies to a panel of pathogens, including mouse hepatitis virus, Sendai virus, and mycoplasma pulmonis.

Tumors. L1210 leukemia was maintained by weekly ip transplants of 10^6 cells/mouse, in DBA/2 mice, according to Geran protocols.[48] For experiments, CD2F1 mice were injected iv with 10^5 cells/mice. The human solid tumors were transplanted subcutaneously on athymic mice using 15−20 mg of tumor brei or maintained in vitro as continuos cultures. For drug testing, fragments of tumors or 5×10^6 (A2780, HCT-116 and DU 145) cells/mouse were implanted into the left flank of recipient mice. When the tumor was palpable (0.2−0.3 g), animals were divided randomly into test groups consisting at least of six mice each (day 0).

Drug Administration and Testing. All drugs solutions were prepared immediately before use. Drug was administered iv in a volume of 10 mg/kg of body weight, according to the indicated schedules. Drug activity for leukemia models was calculated as % of median survival time (T/C%) of treated animals compared to the control group. T/C% was determined by comparing the median survival time (MST) of the treated group with that of the control group where: T/C % = [(MST of treated mice/MST of controls) × 100]. In experiments with solid tumors, the activity was evaluated as % inhibition of tumor growth (%TI) one week after the last treatment. Tumor growth was assessed by caliper: the two diameters were recorded, and the tumor weight was calculated according to the following formula: length × $(\text{width})^2/2$. The %TI was calculated according to the equation: %TI = 100 − (mean tumor weight of treated group/mean tumor weight of control group × 100). Toxicity was evaluated on the basis of weight loss and the gross autopsy findings, mainly in terms of reduction of spleen and liver size.

Measurement of GSH Level and GST Activity. Total GSH was measured from cell growing in culture as previously described.[42] Total GST activity was determined using CDNB as substrate.[49] Reaction was performed using cytosolic extracts and measuring the conversion of CDNB by GST using a spectrophotometer. The data are normalized for the amount of proteins present in each sample.

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Supporting Information Available: Further experimental details concerning the synthesis of intermediates are available free of charge via the Internet at http:/pubs.acs.org.

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